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(54) Title: NUCLEIC ACID ENCODING *M. TUBERCULOSIS* ALGU PROTEIN

(57) Abstract

The invention relates to *Mycobacterium tuberculosis* RNA polymerase algU sigma subunit protein, DNA encoding, and methods of detecting inhibitors of the RNA polymerase.

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NUCLEIC ACID ENCODING *M. TUBERCULOSIS* ALGU PROTEIN

Background of the Invention

Mycobacteria are gram-positive bacilli, nonmotile rod-shaped organisms that do not form spores. The composition of the cell wall includes a very high concentration of lipids complexed to a variety of peptides and polysaccharides. The unusual structure of the cell wall distinguishes mycobacteria from most other bacteria and is detectable by its resistance to acid-alcohol staining.

The disease caused by *M. tuberculosis* is a progressive, deadly illness that tends to develop slowly and follows a chronic course (Plorde, 1994). It is presently estimated that one-third of the world's population is infected with *M. tuberculosis*, 30 million of whom have active disease (Plorde, 1994). An additional 8 million people develop the disease annually (Plorde, 1994). Most infections are caused by inhalation of droplet nuclei carrying the mycobacterium. A single cough can generate 3000 infected droplet nuclei and even 10 bacilli may be sufficient to cause a pulmonary infection. In addition to the primary infection, reactivation of the disease can occur in older people and in immunocompromised patients.

When intracellular pathogens, such as *Mycobacterium tuberculosis*, are ingested by macrophages the bacteria are under environmental stress. The genes required for survival following uptake by macrophages can provide insight into mycobacterial pathogenesis, and provide novel targets for developing antibacterial agents. The ability to adapt to the intracellular stress requires regulation of complex gene expression and this regulation may

be mediated in part by one or more alternative sigma factors. Therefore stress response alternative sigma factors (sigE family) from *M. tuberculosis* are potential novel targets for antibacterial therapeutics.

Extracellular environmental stress can significantly affect the survival of the bacteria. As part of the adaptive response by the bacteria the alternative sigma factors play a critical role in coordinate regulation of gene expression. For example, survival following extreme temperature in *Escherichia coli* is regulated by a family of alternative sigma factors known as the sigE family (Keiichiro et al., Raina et al., Rouviere et al.). Alginate production in *Pseudomonus aeruginosa* is also regulated by the sigE family member known as the *algU* gene (Deretic et al.). Respiratory infections with mucoid *P. aeruginosa* in cystic fibrosis (CF) patients are the major cause of mortality. Although initial colonizing strains are nonmucoid, the bacteria are converted to mucoid *P. aeruginosa* in the CF lung. This conversion to mucoidy is regulated by the alternative sigma factor *algU* (Martin et al.).

Sigma (σ) factors are positive regulators of general transcription initiation that enhance transcriptional specificity. The basic unit of the eubacterial transcription apparatus is the DNA-dependent RNA polymerase holoenzyme, a complex consisting of five protein subunits: two copies of the α subunit and one copy each of the β, β', and σ subunits. The α, β and β' subunits are invariant in a given bacterial species and together form core RNA polymerase. Open promoter complexes form only when holoenzyme is bound at a promoter (Gross et al., 1992). When the newly synthesized RNA chain is 8-9 nucleotides long, σ factor dissociates from the complex and the elongation process is begun (von Hippel, et al., 1992). After transcription is terminated, σ factor rebinds core polymerase, creating holoenzyme for another round of initiation (von Hippel, et al., 1992). This series of biochemical activities has been termed "the transcription cycle".

Rifampicin, a highly specific inhibitor of mycobacterium/RNA polymerase, is one of the primary drugs of choice for treatment of tuberculosis. Combination treatment with isoniazid is typical if there is no risk of developing multi-drug resistance. Prolonged treatment regimens are necessary and can take up to nine months. Failure to complete the prolonged treatment course is one of the contributing factors in the development of resistant

bacterial strains. Rifabutin is an effective analog of rifampicin, but 70% of rifampicin-resistant strains are also rifabutin-resistant.

Although RNA polymerase is a well-validated target for anti-mycobacterial therapy, discovery of inhibitors of *M. tuberculosis* RNA polymerase is hampered by a lack of information concerning components of the *M. tuberculosis* transcriptional apparatus, difficulties in obtaining sufficient yields of active enzymes for biochemical studies, and technical and biosafety concerns surrounding the handling of live cultures of *M. tuberculosis*. Establishment of an *in vitro* transcription system employing purified and reconstituted RNA polymerase would greatly advance efforts to identify new therapeutic agents active against tuberculosis. It is very possible that molecules that inhibit σ functions may not affect eukaryotic general transcription. Thus, σ factors are a reasonable target for development of transcriptional inhibitors. Therefore, molecules that inhibit σ factor function may be used as general transcriptional inhibitors and antibacterial therapeutics.

Accordingly, there is a need in the art for compositions and methods utilizing cloned genes and purified proteins derived from *M. tuberculosis* RNA polymerase.

Summary of the Invention

The present invention is based on the isolation and characterization of DNA encoding the σ subunit of RNA polymerase derived from the *algU* gene from *M. tuberculosis*. In one aspect, the invention provides a purified, isolated nucleic acid having the sequence shown in Figure 3. The invention also encompasses sequence-conservative and function-conservative variants of this sequence. The invention also provides vectors comprising these sequences, and cells comprising the vectors.

In another aspect, the present invention provides a purified, isolated polypeptide encoded by the nucleic acid sequence shown in Figure 3, as well as function-conservative variants thereof.

In yet another aspect, the invention provides *in vitro* methods for high-throughput screening to detect inhibitors of *M. tuberculosis* RNA polymerase. The methods are carried out by the steps of:

a) providing a mixture comprising

(i) purified *M. tuberculosis* RNA polymerase containing the *algU* σ factor and

(ii) a DNA template encoding a promoter sequence that is recognized by *M. tuberculosis* RNA polymerase containing the *algU* subunit;

b) incubating the mixture in the presence of test compounds to form test samples, and in the absence of test compounds to form control samples, under conditions that result in RNA synthesis in the control samples;

c) measuring RNA synthesis in the test and control samples; and

d) comparing the RNA synthesis detected in step (c) between the test and control samples. According to the invention, an RNA polymerase inhibitor is a test compound that causes a reduction in RNA synthesis measured in the test sample relative to RNA synthesis measured in the control sample.

In yet another aspect, the invention provides *in vivo* methods for high-throughput screening to detect inhibitors of *M. tuberculosis* RNA polymerase. The methods are carried out by the steps of:

a) providing a non-mycobacterial bacterial strain, preferably E. coli, that

(i) has been transformed with a DNA template encoding a promoter sequence that is recognized by *M. tuberculosis* RNA polymerase containing the *algU* subunit, and

(ii) expresses enzymatically active *M. tuberculosis* RNA polymerase (e.g., α, β, β' plus the *algU* σ subunit disclosed herein);

b) incubating the bacterial strain of (a) in the presence of test compounds to form test samples, and in the absence of test compounds to form control samples;

c) measuring RNA synthesis in the test and control samples; and

d) comparing the RNA synthesis detected in step (c) between the test and control samples. According to the invention, an RNA polymerase inhibitor is a test compound that causes a reduction in RNA synthesis measured in the test sample relative to RNA synthesis measured in the control sample.

These and other aspects of the present invention will be apparent to those of ordinary skill in the art in light of the present specification and appended claims.

Description of the Drawings

Figure 1. PCR amplification of *M. tuberculosis* H37Rv genomic DNA. lane M: DNA marker (123bp, Gibco-BRL), lane 1: primer P1 only, lane 2: primer P2 only and lane 3: primer P1 and P2. The amplified DNA fragment (arrow in Figure 1) was gel purified and subcloned into pCRScript (Stratagene) plasmid.

Figure 2A. Southern blot analysis of *M. tuberculosis* H37Rv DNA and cosmid clones. **A.** *M. tuberculosis* H37Rv genomic DNA were digested with restriction enzymes: BamH I (lane 1), Pst I (lane 2), Pvu II (lane 3), Sma I (lane 4) and Xmn I (lane5) and analyzed by Southern hybridization using the PCR amplified DNA fragment as a probe. Sizes of DNA markers (^{35}S-DNA Marker, Amersham) are indicated in kb.
2B. Two different positive clones (designated 2D11 and 4D11) isolated from an *M. tuberculosis* cosmid library were digested with BamH I (lane 1 and 4), Pvu II (lane 2 and 5) and Sma I (lane 3 and 6) and hybridized with the PCR-generated sigma gene as a probe.

Fig. 3. Nucleotide and deduced amino acid sequences of the *M. tuberculosis* H37Rv *algU* gene.

Fig. 4. Alignment of the inferred amino acid sequence of the *M. tuberculosis* (Mt) H37Rv *algU* gene with sequences of extracellular function family of sigma subunits from other bacteria (*Streptomyces coelicolor, Pseudomonas aeruginosa , Escherechia coli* and *Hemophilus influenzae*) Shading indicates identical amino acid residues. Amino acid sequence alignments were performed using MegAlign (DNAStar).

Detailed Discussion of the Invention

All patents, patent applications and literature references cited herein are hereby incorporated in their entirety. In the case of inconsistencies, the present disclosure will prevail.

The present invention is based on the isolation of a fragment of the *M. tuberculosis algU* gene, encoding an alternative σ subunit of RNA polymerase. As described

in Example 1 below. PCR amplification of *M. tuberculosis* genomic DNA with primers based on the *M. leprae algU* DNA sequence generated an expected size of DNA (180 base pairs) (Figure 1). The PCR amplified DNA had >90% identity to the *M. leprae* gene. Southern blot analysis demonstrated the presence of a single copy of this gene in *M. tuberculosis* (Figure 2A). The amplified DNA was utilized as a hybridization probe to recover the entire *algU* gene from a cosmid library of genomic DNA from virulent *M. tuberculosis* strain H37RV. Nucleotide sequencing indicated that the 675 bp *M. tuberculosis algU* open reading frame (ORF) encodes a protein of 24.3 kDa (225 amino acids) which shows significant structural similarity to the σ subunits of diverse bacterial species with greatest identity to the stress related extracellular function family of σ subunits of *Streptomyces coelicolor*, *Pseudomonas aeruginosa*, *Escherochia coli* and *Hemophius influenzae*. The sigma factors from *S. coelicolor* and *P. aeruginosa* , *E. coli* and *H. influenzae* are 24%, 20%, 21% and 16% identical to the *M. tuberculosis* sequence respectively (Figure 4).

The *P. aeruginosa algU* gene is part of a large operon that contains genes for anti-sigma factors (*mucA* and *mucB*) and a protease (*mucD*) (Schurr et al.). Further nucletodide sequencing and availability of an integrated map of the genome of *M. tuberculosis* H37Rv (Philipp et al., 1996) is expected to clarify the structural organization and position of the *algU* locus of *M. tuberculosis*.

In practicing the present invention, many techniques in molecular biology, microbiology, recombinant DNA, and protein biochemistry such as these explained fully in, for example, Sambrook *et al.*, 1989, *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York; *DNA Cloning: A Practical Approach*, Volumes I and II, 1985 (D.N. Glover ed.); *Oligonucleotide Synthesis*, 1984, (M.L. Gait ed.); *Transcription and Translation*, 1984 (Hames and Higgins eds.); *A Practical Guide to Molecular Cloning*; the series, *Methods in Enzymology* (Academic Press, Inc.); and *Protein Purification: Principles and Practice*, Second Edition (Springer-Verlag, N.Y.), may be used.

The present invention encompasses nucleic acid sequences encoding the *algU* gene of *M. tuberculosis*, enzymatically active fragments derived therefrom, and related sequences. As used herein, a nucleic acid that is "derived from" a sequence refers to a nucleic

acid sequence that corresponds to a region of the sequence, sequences that are homologous or complementary to the sequence, and "sequence-conservative variants" and "function-conservative variants". Sequence-conservative variants are those in which a change of one or more nucleotides in a given codon position results in no alteration in the amino acid encoded at that position. Function-conservative variants are those in which a given amino acid residue in the *algU* subunit has been changed without altering the overall conformation and function of the polypeptide, including, but not limited to, replacement of an amino acid with one having similar physico-chemical properties (such as, for example, acidic, basic, hydrophobic, and the like). Fragments of the *algU* subunit that retain enzymatic activity can be identified according to the methods described herein, e.g., expression in *E. coli* followed by enzymatic assay of the cell extract.

The nucleic acids of the present invention include purine- and pyrimidine-containing polymers of any length, either polyribonucleotides or polydeoxyribonucleotides or mixed polyribo-polydeoxyribo nucleotides. This includes single- and double-stranded molecules, i.e., DNA-DNA, DNA-RNA and RNA-RNA hybrids, as well as "protein nucleic acids" (PNA) formed by conjugating bases to an amino acid backbone. This also includes nucleic acids containing modified bases. The nucleic acids may be isolated directly from cells. Alternatively, PCR can be used to produce the nucleic acids of the invention, using either chemically synthesized strands or genomic material as templates. Primers used for PCR can be synthesized using the sequence information provided herein and can further be designed to introduce appropriate new restriction sites, if desirable, to facilitate incorporation into a given vector for recombinant expression.

The nucleic acids of the present invention may be flanked by natural *M. tuberculosis* regulatory sequences, or may be associated with heterologous sequences, including promoters, enhancers, response elements, signal sequences, polyadenylation sequences, introns, 5'- and 3'- noncoding regions, and the like. The nucleic acids may also be modified by many means known in the art. Non-limiting examples of such modifications include methylation, "caps", substitution of one or more of the naturally occurring nucleotides with an analog, and internucleotide modifications such as, for example, those with uncharged linkages (e.g., methyl phosphonates, phosphotriesters, phosphoroamidates, carbamates, etc.) and with charged

linkages (e.g., phosphorothioates, phosphorodithioates, etc.). Nucleic acids may contain one or more additional covalently linked moieties, such as, for example, proteins (e.g., nucleases, toxins, antibodies, signal peptides, poly-L-lysine, etc.), intercalators (e.g., acridine, psoralen, etc.), chelators (e.g., metals, radioactive metals, iron, oxidative metals, etc.), and alkylators. The nucleic acid may be derivatized by formation of a methyl or ethyl phosphotriester or an alkyl phosphoramidate linkage. Furthermore, the nucleic acid sequences of the present invention may also be modified with a label capable of providing a detectable signal, either directly or indirectly. Exemplary labels include radioisotopes, fluorescent molecules, biotin, and the like.

The invention also provides nucleic acid vectors comprising the disclosed *algU* subunit sequences or derivatives or fragments thereof. A large number of vectors, including plasmid and fungal vectors, have been described for replication and/or expression in a variety of eukaryotic and prokaryotic hosts. Non-limiting examples include pKK plasmids (Clontech), pUC plasmids, pET plasmids (Novagen, Inc., Madison, WI), or pRSET or pREP (Invitrogen, San Diego, CA), and many appropriate host cells, using methods disclosed or cited herein or otherwise known to those skilled in the relevant art. Recombinant cloning vectors will often include one or more replication systems for cloning or expression, one or more markers for selection in the host, e.g. antibiotic resistance, and one or more expression cassettes. Suitable host cells may be transformed/transfected/infected as appropriate by any suitable method including electroporation, $CaCl_2$ mediated DNA uptake, fungal infection, microinjection, microprojectile, or other established methods.

Appropriate host cells include bacteria, archebacteria, fungi, especially yeast, and plant and animal cells, especially mammalian cells. Of particular interest are *E. coli, B. subtilis, Saccharomyces cerevisiae, Saccharomyces carlsbergensis, Schizosaccharomyces pombe, SF9* cells, C129 cells, 293 cells, *Neurospora*, and CHO cells, COS cells, HeLa cells, and immortalized mammalian myeloid and lymphoid cell lines. Preferred replication systems include M13, ColE1, SV40, baculovirus, lambda, adenovirus, and the like. A large number of transcription initiation and termination regulatory regions have been isolated and shown to be effective in the transcription and translation of heterologous proteins in the various hosts. Examples of these regions, methods of isolation, manner of manipulation, etc. are known in

the art. Under appropriate expression conditions, host cells can be used as a source of recombinantly produced Mycobacterial-derived peptides and polypeptides.

Advantageously, vectors may also include a transcription regulatory element (i.e., a promoter) operably linked to the *algU* subunit portion. The promoter may optionally contain operator portions and/or ribosome binding sites. Non-limiting examples of bacterial promoters compatible with *E. coli* include: *trc* promoter, β-lactamase (penicillinase) promoter; lactose promoter; tryptophan (trp) promoter; arabinose BAD operon promoter; lambda-derived Pl promoter and N gene ribosome binding site; and the hybrid tac promoter derived from sequences of the trp and lac UV5 promoters. Non-limiting examples of yeast promoters include 3-phosphoglycerate kinase promoter, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) promoter, galactokinase (GALI) promoter, galactoepimerase promoter, and alcohol dehydrogenase (ADH) promoter. Suitable promoters for mammalian cells include without limitation viral promoters such as that from Simian Virus 40 (SV40), Rous sarcoma virus (RSV), adenovirus (ADV), and bovine papilloma virus (BPV). Mammalian cells may also require terminator sequences and poly A addition sequences, and enhancer sequences which increase expression may also be included. Sequences which cause amplification of the gene may also be desirable. Furthermore, sequences that facilitate secretion of the recombinant product from cells, including, but not limited to, bacteria, yeast, and animal cells, such as secretory signal sequences and/or prohormone pro region sequences, may also be included.

Nucleic acids encoding wild-type or variant subunit polypeptides may also be introduced into cells by recombination events. For example, such a sequence can be introduced into a cell, and thereby effect homologous recombination at the site of an endogenous gene or a sequence with substantial identity to the gene. Other recombination-based methods, such as non-homologous recombinations or deletion of endogenous genes by homologous recombination, may also be used.

algU subunit-derived polypeptides according to the present invention, including function-conservative variants, may be isolated from wild-type or mutant *M. tuberculosis* cells, or from heterologous organisms or cells (including, but not limited to, bacteria, fungi, insect, plant, and mammalian cells) into which a subunit-derived protein-coding sequence has been introduced and expressed. Furthermore, the polypeptides may be part of recombinant fusion

proteins. Alternatively, polypeptides may be chemically synthesized by commercially available automated procedures, including, without limitation, exclusive solid phase synthesis, partial solid phase methods, fragment condensation or classical solution synthesis.

"Purification" of a σ subunit polypeptide refers to the isolation of the polypeptide in a form that allows its enzymatic activity to be measured without interference by other components of the cell in which the polypeptide is expressed. Methods for polypeptide purification are well-known in the art, including, without limitation, preparative disc-gel electrophoresis, isoelectric focusing, HPLC, reversed-phase HPLC, gel filtration, ion exchange and partition chromatography, and countercurrent distribution. For some purposes, it is preferable to produce the polypeptide in a recombinant system in which the protein contains an additional sequence tag that facilitates purification, such as, but not limited to, a polyhistidine sequence. The polypeptide can then be purified from a crude lysate of the host cell by chromatography on an appropriate solid-phase matrix. Alternatively, antibodies produced against the σ subunit or against peptides derived therefrom can be used as purification reagents. Other purification methods are possible.

The isolated polypeptides may be modified by, for example, phosphorylation, sulfation, acylation, or other protein modifications. They may also be modified with a label capable of providing a detectable signal, either directly or indirectly, including, but not limited to, radioisotopes and fluorescent compounds.

Screening Methods to Identify Anti-tuberculosis Agents

The methods and compositions of the present invention can be used to identify compounds that inhibit the function of *M. tuberculosis* RNA polymerase and thus are useful as anti-tuberculosis agents. This is achieved by providing active recombinant *algU* subunit according to the present invention, in combination with other components of RNA polymerase, in a context in which the inhibitory effects of test compounds can be measured.

In a preferred embodiment, recombinant *M. tuberculosis* RNA polymerase subunits (α, β, β' plus the σ subunit disclosed herein) are purified in milligram quantities from *E. coli* cultures by affinity methods utilizing a hexahistidine tagged α and σ subunits. Enzymatically active holoenzyme is reconstituted using these components. The active

polymerase is then incubated in the presence of test compounds to form test mixtures, and in the absence of test compounds to form control mixtures. *In vitro* transcription is then carried out using a DNA template containing appropriate promoter and reporter sequences. (See Example 3 below.)

In another embodiment, *M. tuberculosis* RNA polymerase subunits (α, β, β' plus the σ subunit disclosed herein) are co-expressed in *E. coli* or another surrogate bacterial cell, in conjunction with an appropriate promoter-reporter gene. The ability of test compounds to differentially inhibit *M. tuberculosis* RNA polymerase is then assessed.

M. tuberculosis promoters useful in practicing the invention include without limitation: hsp 60 promoter (Stover et al., 1991); cpn-60 promoter (Kong et al., 1993); 85A antigen promoter (Kremer, 1995); PAN promoter (Murray et al., 1992); 16S RNA promoter (Ji et al., 1994); and askβ promoter (Cirillo et al., 1994). Useful reporter genes include without limitation xylE (Curcic et al., 1994); CAT (Das Gupta et al., 1993); luciferase (Cooksey et al., 1993); green fluorescent protein (Dhadayuthap et al., 1995); and lacZ (Silhavy et al., 1985).

It will be understood that the present invention encompasses *M. tuberculosis* RNA polymerases containing the *algU* σ factor disclosed herein, which is used in conjunction with particular promoters that are recognized by RNA polymerase containing this σ factor. The invention also encompasses the identification of additional promoters that are recognized by the particular σ subunit of the present invention. This is achieved by providing a library of random *M. tuberculosis* gene fragments cloned upstream of an appropriate reporter gene (see above). The library is transformed into *M. tuberculosis* or *M. smegmatis* and reporter gene expression is measured. Alternatively, the library is transformed into another bacterial cell, such as, e.g., *E. coli*, which expresses *M. tuberculosis* RNA polymerase core subunits as well as the σ subunit of the present invention and cognate promoters that drive reporter gene expression. In yet another embodiment, expression of an *M. tuberculosis* σ factor confers new recognition properties on *E. coli* RNA polymerase and permits isolation of promoters utilized specifically by a particular *M. tuberculosis* σ subunit.

Preferably, both *in vitro* and *in vivo* screening methods of the present invention are adapted to a high-throughput format, allowing a multiplicity of compounds to be tested in

a single assay. Such inhibitory compounds may be found in, for example, natural product libraries, fermentation libraries (encompassing plants and microorganisms), combinatorial libraries, compound files, and synthetic compound libraries. For example, synthetic compound libraries are commercially available from Maybridge Chemical Co. (Trevillet, Cornwall, UK), Comgenex (Princeton, NJ), Brandon Associates (Merrimack, NH), and Microsource (New Milford, CT). A rare chemical library is available from Aldrich Chemical Company, Inc. (Milwaukee, WI). Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant and animal extracts are available from, for example, Pan Laboratories (Bothell, WA) or MycoSearch (NC), or are readily producible. Additionally, natural and synthetically produced libraries and compounds are readily modified through conventional chemical, physical, and biochemical means (Blondelle et al., *TibTech* 14:60, 1996). preferably using automated equipment, to allow for the simultaneous screening of a multiplicity of test compounds.

Useful anti-tuberculosis compounds are identified as those test compounds that decrease tuberculosis-specific transcription. Once a compound has been identified by the methods of the present invention as an RNA polymerase inhibitor, *in vivo* and *in vitro* tests may be performed to further characterize the nature and mechanism of the inhibitory activity. For example, classical enzyme kinetic plots can be used to distinguish, e.g., competitive and non-competitive inhibitors.

Compounds identified as RNA polymerase inhibitors using the methods of the present invention may be modified to enhance potency, efficacy, uptake, stability, and suitability for use in pharmaceutical formulations, etc. These modifications are achieved and tested using methods well-known in the art.

The present invention is further described in the following examples which are intended to further describe the invention without limiting the scope thereof.

Example 1

In the present Example, the following Materials and Methods were used.

PCR amplification: Based on the *M. leprae* cosmid sequence (cosmid B-1620, Genbank accession #U-00015, position 36121-35942), a set of primers was designed and the

sequence of these primers was: 5'-ATGAACGAACTGCTCGAGATCTTGCCTGCC-3' (P1) and 5'-TCACCCGCCGCGACGATCTCGGACGTCAAC-3'(P2). Amplification was performed using 100ng of *M. tuberculosis* H37Rv genomic DNA using a programmable thermal controller (PTC100, MJ Research, Inc.). The PCR conditions were as follows: reaction volume 100µl; *pfu* cloned DNA polymerase (Stratagene); 0.2 mM dNTPs (Boehringer-Mannheim); 100ng of primer ; one cycle of 94°C for 1 minute, thirty cycle of 94°C for one minute, 50°C for one minute and 72°C for one minute.

Southern blot analysis: Restriction enzyme digests of *M. tuberculosis* H37Rv chromosomal DNAs were electrophoresed on 1 % TAE-agarose gels and transferred to nytran membranes (Schleicher and Schuell) using a Pressure Blotter (Stratagene). Probe labeling was performed using the rediprime DNA labelling system (Amersham) essentially as described by the supplier. Hybridization was performed using 6xSSC, 5xDenhardt solution, 0.5% SDS, 0.1 mg per ml Salmon Sperm DNA and 50% formamide. Washing was performed using 2xSSC, 0.5%SDS, at room temperature for 15 min and 0.1xSSC, 0.5% SDS at 37°C for 15 min.

Cosmid hybridizations: A transducing lysate of a cosmid library of *M. tuberculosis* H37Rv genomic DNA in vector pYA3060 was generously supplied by Dr. J. Clark-Curtiss. Cosmid-bearing *E. coli* χ2819T (Jacobs et al, 1986) colonies representing roughly five genomic equivalents were individually picked to wells of sterile 96-well microtiter dishes and propagated at 30°C in Luria broth containing ampicillin at 30 µg/ml and thymidine at 50 µg/ml. Colonies were grown overnight at room temperature on the above media as nylon filter replicas of the library. Filters were processed for colony hybridization by standard methods and probe hybridizations performed as described above. Cosmid DNAs were purified using maxiprep columns (Qiagen).

DNA sequencing and analysis: Plasmid templates for nucleotide sequencing were purified using maxiprep columns (Qiagen). PCR cycle sequencing (ABI Prizm) was carried out with an Applied Biosystems automated sequencer at the Massachusetts General Hospital DNA Sequencing Core Facility, Department of Molecular Biology (Boston, MA).

(a) Cloning of *M. tuberculosis algU* gene

A DNA fragment (180base pair) that contains the *M. tuberculosis algU* gene was identified by using PCR amplification of *M. tuberculosis* H37Rv genomic DNA with primers that were derived from the *M. leprae* cosmid sequence. To determined whether the amplified DNA fragment contains the *algU* gene, the 180 base pair DNA fragment was subcloned into a pCRScript (Stratagene) plasmid and nucleotide sequences were determined. The deduced amino acid sequence of the PCR product showed significant homology to the *algU* sequence from other bacteria (Figure 4).

(b) Southern blot analysis and isolation of the full length *M. tuberculosis algU* gene

To see whether the cloned *algU* gene is a single copy of gene in *M. tuberculosis* Southern blot analysis was performed. The PCR cloned DNA fragment was used as a probe to analyzed the *M. tuberculosis* H37Rv genomic DNA that was digested with endonucleases. The PCR cloned DNA probe recognized a single band in each digested chromosomal DNA (Figure 2), and it was concluded that the *algU* gene is a single copy of gene in *M. tuberculosis*.

The full-length *algU* gene was obtained from a cosmid library of *M. tuberculosis* H37Rv genomic fragments (kindly provided by Dr. J. Clark-Curtiss) using the 180 bp as a probe. Screening of 552 cosmid-bearing *E. coli* colonies (representing roughly 5 genome equivalents) with the *algU* gene fragment yielded 5 positive clones. One *algU*-hybridizing cosmid clone, 4D11 was analyzed, and Southern blotting of 4D11 DNA digested with a panel of restriction enzymes confirmed that the no gross structural rearrangements of the *algU* gene had occurred during cloning (Fig. 3). The 1.1kb BamH I, 1.2 kb PvuII and 1 kb Sma I *algU*-hybridizing fragments of cosmid 4D11 were subcloned into vector pSKII+ prior to nucleotide sequencing.

(c) Sequence analysis of the *M. tuberculosis algU* gene

Nucleotide sequencing was performed on plasmid subclones shown in Figure 4. The sequence encodes a 675 bp ORF which has an overall G+C composition of 63 % (85 % for bases occupying the codon third position). Assuming that the ATG at position 53-55 serves as the initiator codon, the ORF is expected to encode a protein of 225 amino acids. A

strong match with the consensus sequence for an *M. tuberculosis* ribosome binding site (CAGGTG), (Novick, 1996) is positioned just upstream of the putative ATG codon. Examination of more than 63 bp of nucleotide sequence upstream of the translation start site did not reveal regions of exact identity with prokaryotic promoter sites. Among σ subunits studied in other bacterial species, the deduced amino acid sequence of the 225 residue *M. tuberculosis* protein displayed greatest similarity to the stress related extracellular function family of sigma subunits of *Streptomyces coelicolor, Pseudomonas aeruginosa, Escherechia coli* and *Hemophilus influenzae* (Fig. 4).

Example 2

Others have shown that overexpressed *E. coli* RNA polymerase subunits can be reconstituted into an enzymatically active protein (Zalenskaya et al., 1990; Kashlev et al., 1993; Tang et al., 1995). The *M. tuberculosis rpoA* (Healy et al.), *rpoB* and *rpoC* genes (Miller et al., 1994) have been cloned and characterized. Using the overexpressed *M. tuberculosis* RNA polymerase subunits, the *in vitro* reconstitution assay to form the enzymatically active core enzyme will be performed. Holoenzyme that contains *algU* sigma subunit can be obtained and biochemical analysis of gene regulation in *M. tuberculosis* will be studied. Transcription inhibitors that act against the holoenzyme that contains the stress related sigma factor will be identified.

Example 3

High Throughput Screens For Inhibitors of M. Tuberculosis RNA Polymerase and σ Subunit

High-throughput screens for anti-tuberculosis agents may be performed using either an *in vitro* or *in vivo* format. In either case, the ability of test compounds to inhibit *M. tuberculosis* RNA polymerase-driven transcription of *M. tuberculosis* promoters is tested.

The *algU* sigma factor of the present invention regulates transcription of promoters characterized by the sigma promotor consensus sequence: GAACTT-(N16/17)-

TCTgA-N(1-5) (Deretic, et al., 1994; Erickson, et al., 1989; Lipnska, et al., 1988; Martin, et al., 1994; Scharr, et al., 1995). Therefore, this promoter is preferred for use herein.

a) In vitro screens:

The following procedure is used for cell-free high-throughput screening. A Tomtec Quadra 96-well pipetting station is used to add the reaction components to polypropylene 96-well dishes. 5 µl aliquots of test compounds dissolved in DMSO (or DMSO alone as a control) are added to wells. This is followed by 20 µl of the RNA polymerase mixture, which consists of: 10 mM DTT, 200 mM KCl, 10 mM Mg^{+2}, 1.5 µM bovine serum albumin, and 0.25 µg reconstituted RNA polymerase. After allowing the test compound to interact with the RNA polymerase, 25 µl of the DNA/NTP mixture is added, containing: 1 µg template DNA (see above), 4 µM [α-^{32}P]-UTP, and 400 µM each CTP, ATP, and GTP.

After incubation for 30 min at 25°C, the reaction is stopped by addition of 150 µl 10% trichloroacetic acid (TCA). After incubation at room temperature for 60 min, the TCA-precipitated RNA is adsorbed onto double-thick glass fiber filtermats using a Tomtec cell harvester. The wells of the microtiter plate and the filter are washed twice with 5% TCA and bound radioactivity is determined using a Wallac microbeta 1450 scintillation counter.

Inhibitory activity due to the test compound is calculated according to the formula:

$$\% \text{ inhibition} = \frac{(\text{cpm}_{\text{positive control}} - \text{cpm}_{\text{sample}})}{\text{cpm}_{\text{positive control}}} \times 100$$

where $\text{cpm}_{\text{positive control}}$ represents the average of the cpm in wells that received DMSO alone, and $\text{cpm}_{\text{sample}}$ represents the cpm in the well that received test compound. Compounds that cause at least 50% inhibition are scored as positive "hits" in this assay.

As an additional control, rifampicin is used at a concentration of 30 nM, which results in a 50-75% inhibition of transcription in this assay.

b) In viv screen:

M. tuberculosis RNA polymerase subunits (α, β, β', and the σ subunit disclosed herein) are expressed in *E. coli* under the control of regulatable promoters by transforming *E. coli* with appropriate plasmids. If the σ subunit is expressed, a DNA sequence comprising the sigE promoter described above is also introduced into the cells to serve as a template for *M. tuberculosis*-specific transcription.

In one embodiment, the sigE promoter sequence is linked to a DNA sequence encoding the xylE gene product, catechol 2, 3-dioxygenase (CDO). When expressed in the *E. coli* cell, CDO converts catechol to 2-hydroxymuconic semialdehyde, which has a bright yellow color (having an absorbance maximum at 375 nm) that is easily detected in whole cells or in crude extracts. The substrate for this enzyme is a small aromatic molecule that easily enters the bacterial cytoplasm and does not adversely affect cell viability.

In a high-throughput format, aliquots of bacterial cultures are incubated in the absence or presence of test compounds, and CDO activity is monitored by measuring absorbance at 375 nm following addition of catechol.

c) Specificity:

Compounds that score as positive in either the *in vitro* or *in vivo* assays described above are then tested for their effect on human RNA polymerase II. Those compounds which do not significantly inhibit human RNA polymerase II will be further developed as potential anti-tuberculosis agents.

REFERENCES

Cirillo et al. (1994) Mol.Microbiol. 11:629.

Cooksey et al. (1993) Antimicrob.Agents.Chemother. 37:1348.

Curcic et al. (1994) Mol.Microbiol. 13:1057.

Das Gupta et al. (1993) J.Bacteriol. 175:5186.

Dhadayuthap et al. (1995) Mol.Microbiol. 17:901.

Jacobs, W.R., et al., (1986) *"In vivo* repackaging of recombinant cosmid molecules for analyses of *Salmonella typhimurium, Streptococcus mutants*, and Mycobacterial Genomic libraries". Infect. Immun. 52:101-109.

Ji et al. (1994) Microbiol. 140:2829.

Kong, et al. (1993) Proc.Natl.Acad,Sci.USA 90:2608.

Kremer (1995) J. Bacteriol. 177:642.

Malakooti et al. (199) J.Bacteriol. 177:6854.

Murray et al. (1992) Mol. Micr0biol. 6:3331.

Novick, R. (1996) "Mycobacteria: Growth, Metabolism, and Molecular Biology". Tuberculosis (Little, Brown, and Co., Boston, MA) pp 187-198.

Stover et al., (1991) Nature 351:456

Silhavy et al. (1985) Microbiol.Rev. 49:398.

Tang, H., et al., (1995) "Rapid RNA polymerase genetics: One-day, no-column preparation of reconstituted recombinant *Escherichia coli* RNA polymerase". Proc. Natl. Acad. Sci. USA 92:4902-4906.

Weiden, M. et al., (1996) "Genetics of *M. tuberculosis*". In Tuberculosis (Little, Brown, and Co., Boston, MA) pp 211-222.

Yura and Ishihama (1979) Genetics of bacterial RNA polymerases. Ann. Rev. Genet. 13:59-97.

Vall-Spinosa, A. et al., N. Engl. J. Med. 283:616-621, 1970

Zalenskaya, K. et al., Gene 89:7-12.

Deretic, V., M. Schurr, J. Boucher, and D. Martin. (1994) Conversion *Pseudomonus aeruginosa* mucoidy in cystic fibrosis: environmental stress and regulation of bacterial virulence by alternative sigma factors. *J Bacteriol* **17**: 2773-2780.

Healy, J. H., J. Bodorova, K. Lam, C. Wobbe (1996) The *rpoA* gene of *Mycobacterium tuberculosis*. Submitted for publication.

Kashlev, M., Martin, E., Polyakov, A., Severinov, K., Nikiforov, V., and Goldfarb, A. (1993) Histidine-tagged RNA polymerase: dissection of the transcription cycle using immobilized enzyme. Gene **130:** 9-14.

Keiichiro, H., M. Amemura, H. Nashimoto, H. Shinagawa, and S. Makino. (1995) The *rpoE* gene of *Escherichia coli*, which encodes σ^E, is essential for bacterial growth at high temperature. *J. Bacteriol* **177**:2918-2922.

Martin, D., M. Scurr, M. Mudd, J. Govan, B. Holloway, and V. Deretic (1993) *Proc. Natl. Acad. Sci.* **90**: 8377-8381.

Miller, L., Crawford, J.T., and Shinnick, T.M. (1994) The *rpoB* gene of *Mycobacterium tuberculosis*. Antimicrob. Agents. Chemo. **38:** 805-811.

Philipp, W.J., Poulet, S., Eiglmeier, K., Pascopella, L., Balasubramanian, V., Heym, B., Bergh, S., Bloom, B.R., Jacobs, W.R., and Cole, S.T. (1996) Proc. Natl. Acad. Sci. USA **93:** 3132-3137.

Raina, S., D.Missiakas, and C. Geogopoulos. (1995) The *rpoE* encoding the $\sigma^{E(\sigma^{24})}$ heat shock sigma factor of *Escherichia coli*. *EMBO J* **14**: 1043-1055.

Rouviere, P., A. De Las Penas, C. Lu, K. Rudd, and C. Gross. (1995) *rpoE* the gene encoding the second heat-shock sigma factor, σ^E, in *Escherichia coli*. *EMBO J* **14**: 1032-1042.

Schurr, M.J., H. Yu., J.M. Martinez-salazar, J.C. Boucher and V. Deretic. (1996) Control of *algU*, a member of the σ^E-like family of stress sigma factors, by the negative regulators *mucA* and *mucB* and *Pseudomonas aeruginosa* conversion to mucoidy in cystic fibrosis. *J. Bacteriol* **178**:4997-5004.

Tang, H., Severinov, K., Goldfarb, A., and Ebright, R.H. (1995) Rapid RNA polymerase genetics: One-day, no-column preparation of reconstituted recombinant *Escherichia coli* RNA polymerase. Proc. Natl. Acad. Sci. USA **92:** 4902-4906.

Zalenskaya, K., Lee, J., Gujuluva, C.N., Shin, Y.K., Slutsky, M. and Goldfarb, A. (1990) recombinant RNA polymerase: inducible overexpression, purification, and assembly of *Escherichia coli rpo* gene products. Gene **89:** 7-12.

Deretic, V., M. Schurr, J. Boucher, and D. Martin. (1994) Conversion *Pseudomonus aeruginosa* mucoidy in cystic fibrosis: environmental stress and regulation of bacterial virulence by alternative sigma factors. J Bacteriol. **17**:2773-2780.

Erickson, J.W., and C.A. Gross. (1989) Identification of the sigma subunit of *Escherichia coli* polymerase: a second sigma factor involved in high-temperature gene expression. Genes Dev. **3**:1462-1471.

Lipnska, B., S. Sharma, and C. Georgopoulos. (1988) Sequence analysis and regulation of the *htrA* gene of *Escherichia coli*: a σ^{32}-independent mechanism of heat-inducible transcription. Nucleic Acids Res. **16**:10053-10067.

Martin, D.W., M.J. Schurr, H. Yu, and V. Deretic. (1994) Analysis of promoters controlled by the putative sigma factor AlgU regulating conversion to mucoidy in *Pseudomonas aeruginosa*: relationship to stress response. J. Bacteriol. **176**:6688-6696.

Schurr, M.J., H. Yu, N. Boucher, and V. Deretic. (1995) Multiple promoters and induction by heat shock of the gen encoding the alternative sigma factor AlgU (σ^E) which controls mucoidy in cystic fibrosis isolates of *Pseudomonus aeruginosa*. J. Bacteriol. **177**:5670-5679.

Plorde, J.J., (1994), *Sherris Medical Microbiology*, p 401-415, Ryan, K.J. (ed) Appleton and Lange Press, Norwalk, Ct.

Gross, C.A., Lonetto, M., and Losick, R., (1992) *Transciptional Regulation*, p 129-176, Cold Spring Harbor Laboratory Press, McKnight, S.L. and Yamamoto, K.R. (eds)

von Hippel, P.H., Yager, T.D., Gill, S.C. (1992) p 179-201, *Transciptional Regulation*, Cold Spring Harbor Laboratory Press, McKnight, S.L. and Yamamoto, K.R. (eds)

Claims:

1. An isolated, purified DNA encoding *M. tuberculosis* RNA polymerase *algU* σ subunit.

2. A DNA as defined in claim 1, wherein said DNA has a sequence selected from the group consisting of the sequence shown in Figure 3, sequence-conservative variants thereof, and function-conservative variants thereof.

3. A DNA vector comprising the nucleic acid sequence of claim 2 operably linked to a transcription regulatory element.

4. A cell comprising a DNA vector as defined in claim 3, wherein said cell is selected from the group consisting of bacterial, fungal, plant, insect, and mammalian cells.

5. A cell as defined in claim 4, wherein said cell is a bacterial cell.

6. An isolated purified polypeptide comprising a polypeptide encoded by a DNA as defined in claim 2.

7. A method for high-throughput screening to detect inhibitors of *M. tuberculosis* RNA polymerase, said method comprising:

a) providing a mixture comprising

(i) purified *M. tuberculosis* RNA polymerase containing the *algU* subunit,

(ii) a DNA template encoding a promoter sequence that is recognized by *M. tuberculosis* RNA polymerase containing said *algU* subunit;

b) incubating said mixture in the presence of test compounds to form test samples, and in the absence of test compounds to form control samples, wherein said

incubating is performed under conditions that result in RNA synthesis in the control samples;

c) measuring RNA synthesis in said test and control samples; and

d) comparing the RNA synthesis detected in step (c) between said test and control samples;

wherein an RNA polymerase inhibitor is a test compound that causes a reduction in RNA synthesis measured in said test sample relative to RNA synthesis measured in said control sample.

8. A method for high-throughput screening to detect inhibitors of *M. tuberculosis* RNA polymerase, said method comprising:

a) providing a non-mycobacterial bacterial strain that

(i) has been transformed with a DNA template encoding a promoter sequence that is recognized by *M. tuberculosis* RNA polymerase containing the *algU* subunit, and

(ii) expresses enzymatically active *M. tuberculosis* RNA polymerase, wherein said polymerase comprises α, β, β', and the *algU* σ subunits;

b) incubating the bacterial strain of (a) in the presence of test compounds to form test samples, and in the absence of test compounds to form control samples;

c) measuring RNA synthesis in the test and control samples; and

d) comparing the RNA synthesis detected in step (c) between the test and control samples;

wherein an RNA polymerase inhibitor is a test compound that causes a reduction in RNA synthesis measured in said test sample relative to RNA synthesis measured in said control sample.

FIG. 1

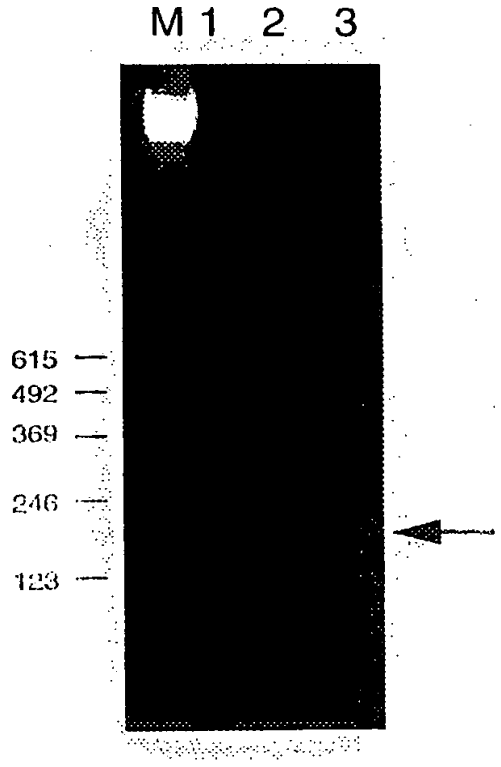

FIG. 2A

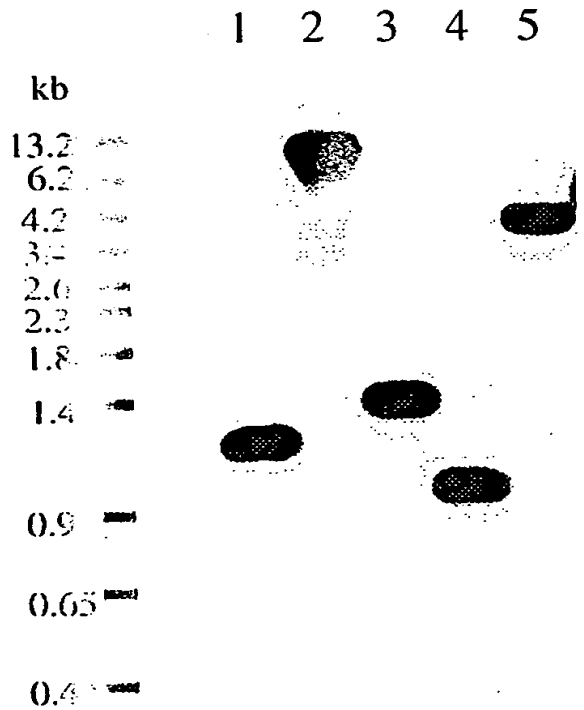

FIG. 2B

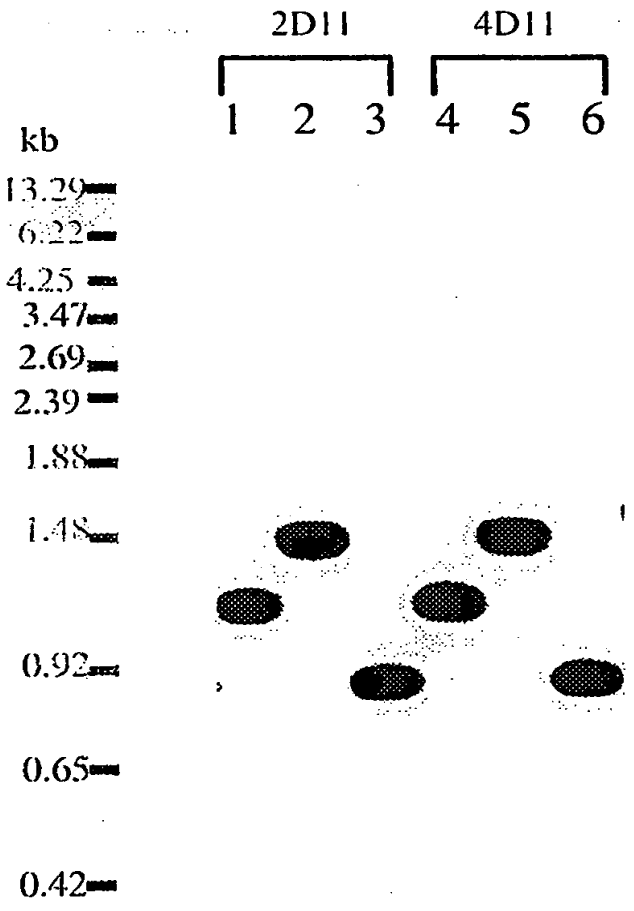

FIG. 3A

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 81 cgttttcagcacaacagctgcgcgaactcaccaaaatacgcaatatttgcgcaggccaggtgcggcgcgccggtgg ATG 159
  1                                                                              M   1

160 CTG GCG TAC CGC TTA AAA CGC GGT TGG GCC GTT ATG GTC GAT CCG GGA GTT AGC CCG GGA 219
  2 L   A   Y   R   L   K   R   G   W   A   V   M   V   D   P   G   V   S   P   G  21

220 TGT GTC CGC TTC GTA ACG TTG GAG ATA TCG CCG TCG ATG ACA ATG CAA GGG GAA CGT CTC 279
 22 C   V   R   F   V   T   L   E   I   S   P   S   M   T   M   Q   G   E   R   L  41

280 GAC GCT GTG GTT GCG GAG GCC GTG GCA GGA GAC CGG AAC GCG CTT CGG GAG GTG CTG GAG 339
 42 D   A   V   V   A   E   A   V   A   G   D   R   N   A   L   R   E   V   L   E  61

340 ACC ATC CGC CCG ATC GTC GTG CGA TAT TGC CGA GCG CGA GTC GGC ACG GTC GAG CGG AGC 399
 62 T   I   R   P   I   V   V   R   Y   C   R   A   R   V   G   T   V   E   R   S  81

400 GGC CTG TCA GCA GAT GAC GTG GCA CAG GAG GTG TGC TTG GCC ACC ATA ACG GCG CTG CCG 459
 82 G   L   S   A   D   D   V   A   Q   E   V   C   L   A   T   I   T   A   L   P 101

460 CGC TAT CGG GAC CGC GGC CGG CCA TTC CTG GCG TTT CTG TAC GGC ATC GCG GCG CAC AAG 519
102 R   Y   R   D   R   G   R   P   F   L   A   F   L   Y   G   I   A   A   H   K 121

520 GTT GCC GAC GCC CAT CGG GCA GCC GGC CGT GAC CGG GCC TAT CCC GCC GAA ACG CTT CCT 579
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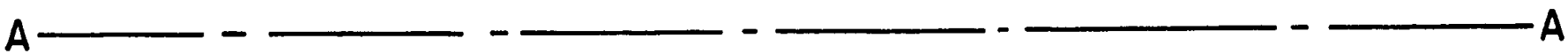
FIG. 3B

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122 V   A   D   A   H   R   A   A   G   R   D   R   A   Y   P   A   E   T   L   P    141

580 GAG CGC TGG TCA GCC GAC GCC GGC CCG GAG CAG ATG GCC ATC GAG GCC GAT TCG GTC ACC  639
142 E   R   W   S   A   D   A   G   P   E   Q   M   A   I   E   A   D   S   V   T    161

640 CGG ATG AAC GAA TTG CTT GAG ATC TTG CCG GCC AAG CAA CGC GAG ATC CTC ATT CTG CGT  699
162 R   M   N   E   L   L   E   I   L   P   A   K   Q   R   E   I   L   I   L   R    181

700 GTT GTC GTC GGC CTG TCC GCG GAA GAG ACC GCC GCC GCC GTC GGC AGC ACC ACG GGG GCG  759
182 V   V   V   G   L   S   A   E   E   T   A   A   A   V   G   S   T   T   G   A    201

760 GTC CGG GTG GCC CAA CAC CGT GCA CTT CAG CGG CTG AAG GAC GAA ATT GTT GCG GCA GGT  819
202 V   R   V   A   Q   H   R   A   L   Q   R   L   K   D   E   I   V   A   A   G    221

820 GAC TAT GCG TGA atttggtaatccccttggcgatcggccgccattggatgagctggcccgcaccgatctgctgctc  895
222 D   Y   A   *                                                                    225

896 gacgcactcgccgaacgggaggaggttgacttcgcggatcctcgcgatgacgcgttggccgccctgctcggacagtggcg  975

976 cgacgacttgaggtggccgccggccagtgccctggtttcacaggacgaggccgtcgccgcgttgcgcgccggggtagcgc 1055

1056 aacggcgacgggctcgtcgcagcctggcggccgtcgggtcggtggccgcg                              1105
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FIG. 4A

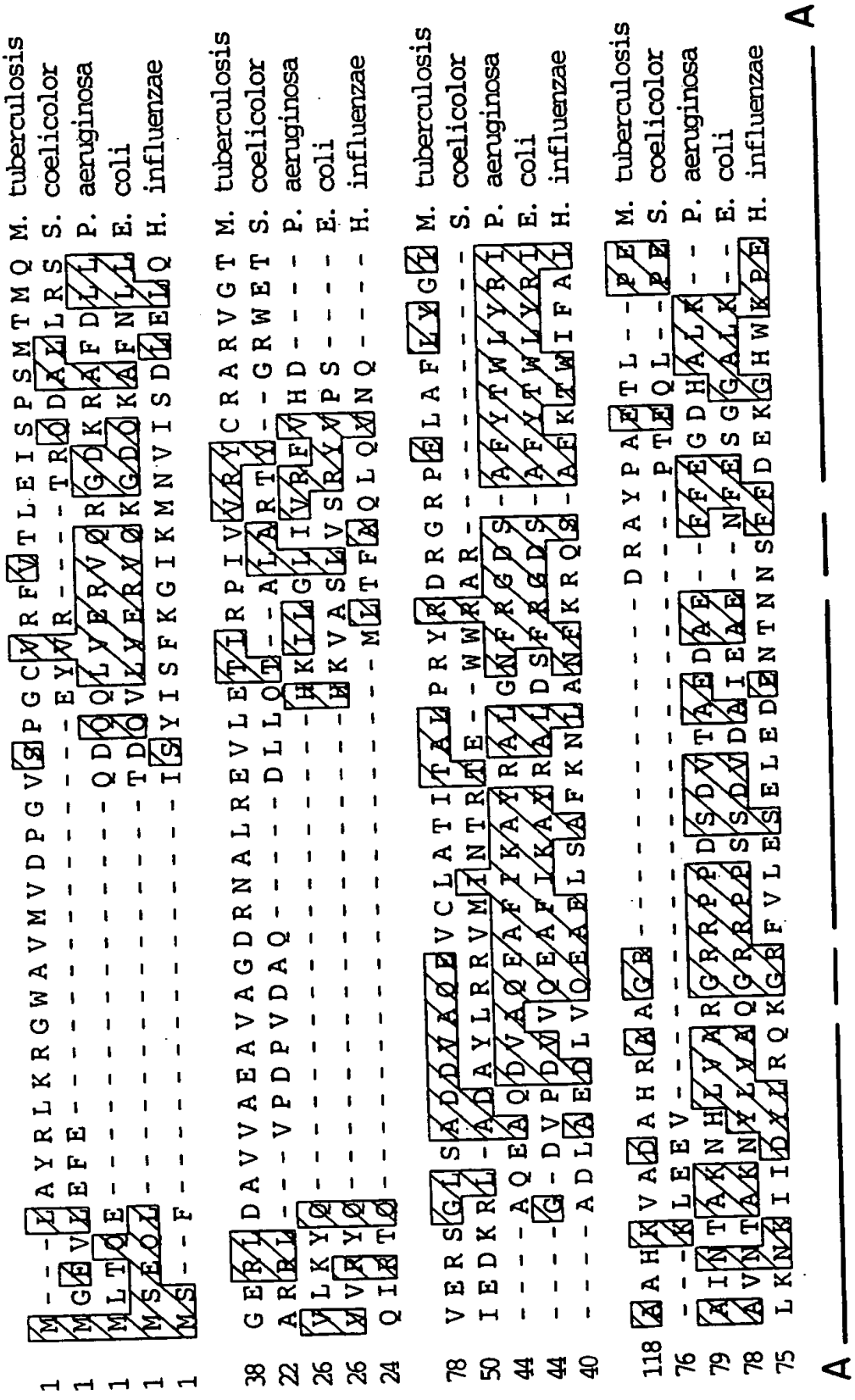
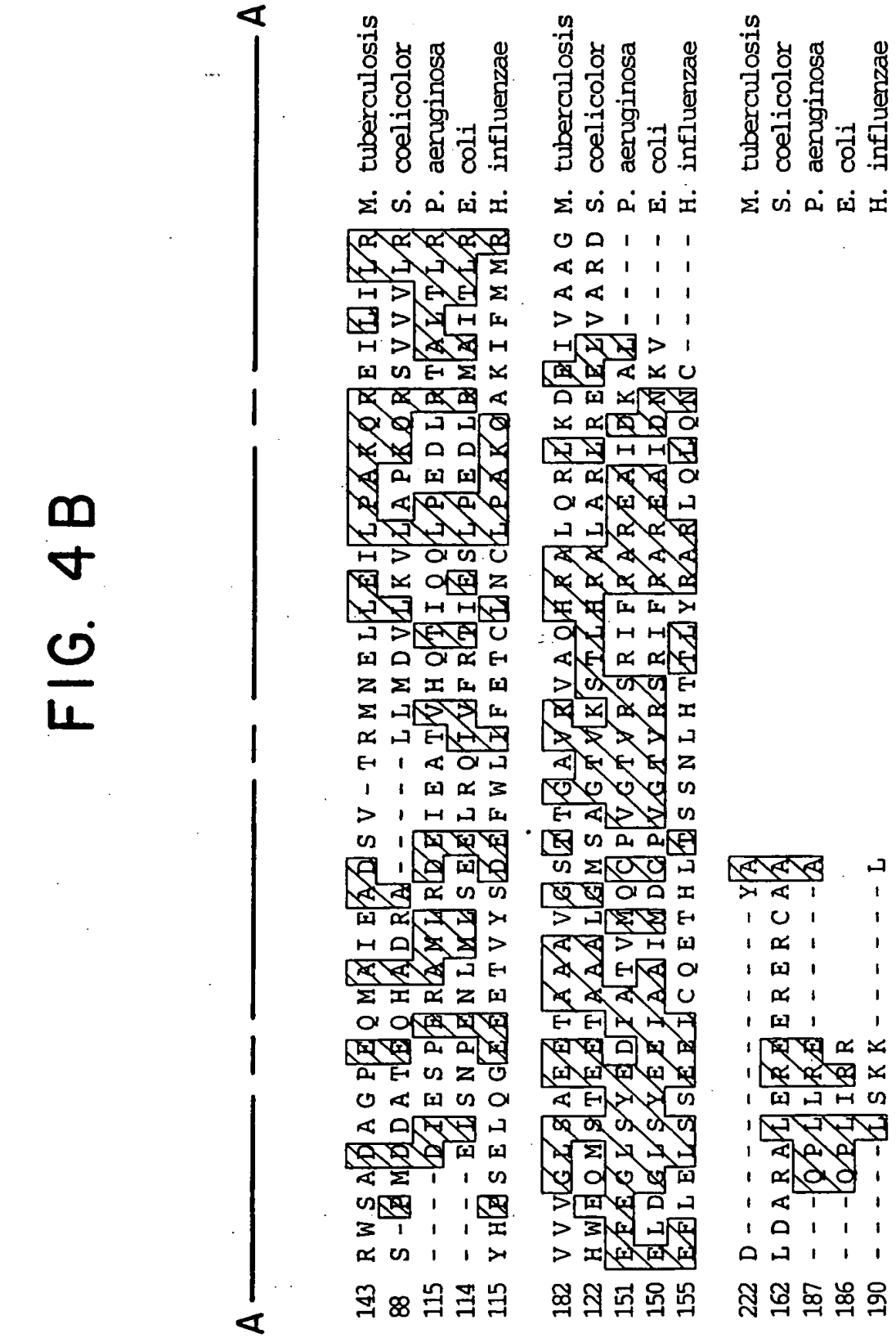


FIG. 4B



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/01244

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12N 9/12, 15/54
US CL :530/350; 435/194, 320.1, 252.3; 536/23.2

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/350; 435/194, 320.1, 252.3; 536/23.2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

none

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DIALOG, APS
search terms: mycobacterium tuberculosis, RNA polymerase, algU, sigma subunit

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	COLLINS et al. Mutation of the principal sigma factor causes loss of virulence in a strain of the Mycobacterium tuberculosis complex. Proceedings of the National Academy of Science. August 1995. Vol. 92. No. 17. pages 8036-8040, see entire document.	1-8
A	LONETTO et al. Analysis of the Streptomyces coelicolor sigE gene reveals the existence of a subfamily of eubacterial RNA polymerase sigma factors involved in the regulation of extracytoplasmic functions. Proceedings of the National Academy of Science. August 1994. Vol. 91, No. 16. pages 7573-7577, see entire document.	1-8

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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"O"	document referring to an oral disclosure, use, exhibition or other means		
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Date of the actual completion of the international search 24 MARCH 1998	Date of mailing of the international search report 14 MAY 1998
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